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**S₁₆ and T₁₈ mannosylation sites of LppX are not essential for its activity in
phthiocerol dimycocerosates localization at the surface of *Mycobacterium
tuberculosis***

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Abstract

LppX is an important virulence factor essential for surface localization of phthiocerol dimycocerosates (DIM) in *Mycobacterium tuberculosis*. Based on Concanavalin A recognition, *M. tuberculosis* LppX (LppX-tb) was initially proposed to be glycosylated in *M. tuberculosis* and more recently this glycosylation was characterized by mass spectrometry analysis on LppX-tb expressed and purified from *Corynebacterium glutamicum*. Here, using this model organism and *Mycobacterium smegmatis*, we show that S16 and T18 residues of LppX-tb are indeed glycosylated with several hexoses units. Interestingly this glycosylation is strictly dependent on the mannosyl transferase PMT which, in *M. tuberculosis*, has been reported to be crucial for virulence. Using a site directed mutagenesis approach, we were able to show that the absence of S16 and T18 glycosylation does not alter phthiocerol dimycocerosates (DIM) localization in *M. tuberculosis*.

Keywords : *Corynebacterium glutamicum*, *Mycobacterium*, glycosylation, phthiocerol dimycocerosates

1. Introduction

Among post-translational modifications of proteins, glycosylation is the most common modification occurring in nature. These modifications which were initially thought to be restricted to eukaryotic cells are now better characterized and known to be widely distributed in bacteria and archaea. All these organisms share similar *N*-glycosylation and *O*-glycosylation process. While *N*-glycosylation corresponds to the addition of glycosyl residues on asparagine, *O*-glycosylation targets serine, threonine or tyrosine. A large variety of glycans (differing in chain length, type of ose, chemical bonds and/or chemical modifications) might be added and thus creating a vast repertoire of modifications contributing to protein diversity [1,2].

In eukaryotes numerous functions have been assigned to protein glycosylation such as protein folding and stability, targeting to distinct cellular compartments and cell to cell recognition [2]. Glycans can affect protein stability and accessibility as well as enable specific interaction. Moreover the nature of the glycan is important as protein dynamics differs depending on glycan chemistry [3]. As an example Prates et al. [3] studied the role of α -mannosylation of a fungal Intrinsically Disordered Protein (IDP) by comparing different glycosylated variants of its linker. Compared to the other tested glycans, α -mannose was shown to increase proteolytic resistance, restrict protein flexibility and lead to more extended conformations of this linker. According to the authors this would explain why, in fungi and yeast, most of the secreted IDP and proteins exhibiting Intrinsically Disordered Regions (IDR) are *O*-mannosylated. In bacteria, the specific need for α -mannose in such structure does not seem essential since their intrinsic high content of proline favors extended conformation of IDR. Still, elongation and further stiffening are observed upon glycosylation of these structures [3].

In recent years there has been a growing interest towards glycosylation in bacteria. Although glycosylation is not restricted to pathogenic bacteria, most studies were performed in these bacteria and conclude to an important role of glycosylation on pathogenicity [1]. Indeed, this phenomenon was described in *Mycobacterium tuberculosis* [4] where the authors demonstrated that disruption of the *M. tuberculosis* Rv1002c gene encoding the membrane-associated protein *O*-mannosyl transferase (PMT) resulted in strong attenuation of the strain. In *M. tuberculosis*, PMT is unique and shown to be responsible for the transfer of mannose residues from polyprenol monophosphate mannose (PPM) to dedicated proteins [5]. In fact, various putatively mannosylated proteins were identified in *M. tuberculosis* by Concanavalin A affinity capture experiments [6]. Several lipoproteins including lipoproteins belonging to the Llp family (Lipid binding lipoprotein) were identified among these proteins including LppX-tb. The gene encoding LppX-tb was initially identified in a transposition mutant of *M. tuberculosis* with an attenuated phenotype [7]. In this mutant, the subcellular distribution of phthiocerol dimycocerosates (DIM) was highly altered as compared to the wild type strain. DIMs are one of the major lipidic virulence factor in pathogenic mycobacteria and their translocation at the surface of the bacterial envelope, essential for the virulence of the strain, is proposed to be mediated by LppX-tb. This hypothesis is quite convincingly corroborated by the structure of LppX-tb which resemble a U-shaped β -half-barrel delimitating a large hydrophobic cavity able to accommodate a DIM molecule [7]. Although supposed to be glycosylated, the complete characterization of LppX-tb modifications has not been so far reported. Indeed, glycosylation of LppX-tb was previously reported during heterologous expression in slow and fast-growing mycobacteria *M. smegmatis*, *M. bovis* and *M. abscessus* [8–10]. In *M. smegmatis* glycosylation was only suggested and not demonstrated. Whereas further results were reported for the two other species for which 1 to 2 hexoses were found associated to the N-

terminal peptide of the protein. However the exact glycosylation site(s) was/were never reported. Similar results have been reported in *Corynebacterium glutamicum*, a model host in which LppX-tb was found to be heterogeneously glycosylated with 1 to 4 hexose(s) units in its N-terminal peptide (aa₆ to aa₂₉) and where α -mannosidase deglycosylation experiments suggested that LppX-tb is indeed mannosylated [11]. Interestingly LppX-tb glycosylation was found to be differently affected by mutations in the lipoprotein post-translational modification pathway in *C. glutamicum* [11,12]. These results enabled the authors to propose that the N-acylation enzyme Lnt associates with PMT but that acylation (S- and N-acylation) is not a prerequisite for glycosylation of LppX-tb in *C. glutamicum*.

As mannosylated LppX-tb is engaged in the virulence process of *M. tuberculosis* we wondered if the attenuation phenotype observed in the PMT mutant [4] resulted from the absence of glycosylation of LppX-tb. For this, we first identified S16 and T18 as the two sites of LppX-tb mannosylation. We then undertook the construction of a variant where both amino acids were modified in order to test the functionality of this LppX-tb variant. Both the glycosylated and non-glycosylated forms of LppX-tb were characterized and found associated to the capsule layer of *M. smegmatis*. In *M. tuberculosis* Mt103, complementation of the knock-out mutant of *LppX-tb* either with a wild or mutated copy of the *LppX-tb* gene fully restored the release of DIM into the culture medium suggesting that glycosylation of S16 and T18 of LppX-tb is not necessary for DIM translocation.

2. Materials and methods

2.1 Bacterial strains and growth conditions

C. glutamicum ATCC13032 RES167 [13] and its derivative *pmt::Km^r*, were routinely cultured on BHI medium (Difco) at 30°C.

Mycobacterium smegmatis MC²155 and *Mycobacterium tuberculosis* Mt103 were grown in Middlebrook 7H9 broth (Fluka) supplemented with Middlebrook ADC enrichment and 0.5% glycerol or BHI. Tween 80 (0.05 %) was added to liquid broth to avoid clumping except for capsule analysis.

2.2. Plasmids

Plasmids pCGL482 [14], pVWEx2 [15], pMV261-Gm (derivative of pMV261) [16] and pVV16 [17] were used as expression vectors in *C. glutamicum*, *M. smegmatis* and *M. tuberculosis* respectively. When appropriate, antibiotics were added at the following concentrations: kanamycin (Km) 25 µg. ml⁻¹, chloramphenicol (Cm) 6 µg. ml⁻¹ for *C. glutamicum* or 30 µg. ml⁻¹ for *E. coli*, gentamycin (Gm) 10 µg. ml⁻¹, hygromycin (Hyg) 25 µg. ml⁻¹ and tetracyclin 10 µg. ml⁻¹.

Cloning of Cg-*pmt* and Ms-*pmt* in *C. glutamicum* : The *pmt* genes of *C. glutamicum* Cg-*pmt* (Cg1014 or NCgl0854) and *M. smegmatis* Ms-*pmt* (MsmeG_5447) [4] were cloned under the tac promoter into the expression vector pVWEx2 [15]. The *pmt* genes were amplified from the genomic DNA of *C. glutamicum* ATCC13032 or *M. smegmatis* mc²155 by PCR using the primers DirCgPmt-*Xba*I and RevCgPmt6His-*Bgl*II or DirMsmPmt-*Xba*I and RevMsmPmt6His-*Bam*HI respectively (Table 1). The PCR products were digested with *Xba*I and *Bgl*II or *Xba*I and

*Bam*HI and cloned into the *Xba*I- and *Bam*HI-digested pVWEx2 resulting in the *pmt*-expressing plasmids pVWEx2-CgPmt-His and pVWEx2-MsPmt-His.

Cloning *lppX*-tb in *M. tuberculosis*: Cloning of *lppX*-tb and *lppX*-tb Δ S16 T18A in the mycobacterial expression vector pVV16 was performed by PCR as described in Sulzenbacher et al. [7] except for the DNA template. Here we used plasmid pMV261 *LppX*-tb and pMV261 *LppX*-tb Δ S16 T18A as templates.

Transformation of the bacterial strains was performed by electrotransformation as described for *E. coli* [18], *C. glutamicum* [19], *M. smegmatis* and *M. tuberculosis* [20].

2.3. *Pmt* gene disruption

The *pmt* gene (Cg1014 or NCgl0854) of *C. glutamicum* ATCC13032 was interrupted by integration of non-replicative plasmid with a kanamycin resistance cassette into the 5' part of the gene. Briefly, a 532 bp-DNA fragment of the *pmt* gene was amplified by PCR using primers PMT1-3 and PMT1-4 (Table 1). The DNA fragment was cloned into the pPrime cloning vector (5PRIME) and used to transform *E. coli* as described by the manufacturer. The recombinant plasmid was transferred into *C. glutamicum* by electroporation and transformants were selected on Km-containing plates. As the plasmid is unable to replicate into *C. glutamicum*, only the bacteria with the plasmid integrated into the genome were able to grow in presence of kanamycin. Correct integration of the plasmid into the *pmt* gene was checked by PCR analysis on the genomic DNA using combinations of primers localized upstream and downstream of the cloned 532 bp-DNA fragment (PMT1-1 and PMT1-6) and into the vector (M13R and M13F(-20)). After sequencing of the PCR products, one clone (*pmt*::*Km^r*) was used for further experiment.

2.4. Site directed mutagenesis of *LppX-tb*

Site-directed mutagenesis of the *lppX-tb* gene was performed by PCR-driven overlap extension [21]. The mutagenic primers and flanking primers used to generate the mutated-*lppX-tb* genes are listed below (Table 1). Plasmid pCGL482 [11] and pMV261 [8] both carrying the native *LppX-tb* gene with a hemagglutinin (HA) and a His₆ epitope fused to the 3' part of the gene were used as templates for PCR. The generated PCR fragments were digested with *EcoRV-XhoI* or *EcoRI* and cloned either into the *EcoRV-XhoI* digested pCGL482-AmyE [11] for *C. glutamicum* or the expression vector pMV261 for *M. smegmatis*. All the mutated genes were sequenced to confirm the mutations. All mutated *lppX-tb* genes contained the hemagglutinin and a His₆ epitope fused to their 3' part.

2.5. Purification of native *LppX-tb* and derivatives

Purification of *LppX-tb* and mutated *LppX-tb* from *C. glutamicum* was performed as described in Mohiman et al. [11].

For purification from *M. smegmatis*, bacteria from liquid cultures were harvested and resuspended in a 50 mM phosphate buffer pH 8.0 containing 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, Sigma) as a protease inhibitor. Cells were broken using a one shot cell disrupter (CellID) at 30kPsi. Unbroken cells were removed by low speed centrifugation (4000 g for 15 min) and the cleared supernatant was centrifuged at 106,000 g for 60 min (rotor Ti 50 Beckman). Total membranes were homogenized in a 50 mM phosphate buffer pH 8.0 containing 4% LDAO (lauryl dimethylamine oxide) and stirred 1h in order to efficiently solubilize membrane proteins. Insoluble material was removed by centrifugation at 106,000 g for 60 min. The soluble membrane proteins were diluted twice and NaCl and imidazole were added to final concentrations of 50 mM and 10

mM respectively in 25 mM phosphate buffer, 2% LDAO, pH 8.0. Ni-NTA agarose previously equilibrated with 25 mM phosphate buffer pH 8.0 with 2% LDAO, 50 mM NaCl and 10 mM imidazole was added to the soluble membrane proteins. The mixture was gently agitated for 1h at 4°C and loaded in an empty column. The agarose resin was washed with ten volumes of 25 mM phosphate buffer pH 8.0 with 1 % LDAO, 50 mM NaCl and 20 mM imidazole to remove weakly associated proteins. LppX-tb was eluted with the same buffer containing 50 mM imidazole. The eluted samples were precipitated with trichloroacetic acid (TCA) 10% to concentrate the proteins. If necessary further purification of LppX-tb was performed with anti HA agarose (Pierce) instead of TCA precipitation.

2.6. Extraction and analysis of proteins from different cellular localisation in C. glutamicum and M. smegmatis

The proteins from the envelope and culture supernatant of *C. glutamicum* were prepared as follows. After growth, cells were separated from the culture supernatant by centrifugation at 7000 g for 10 min. Proteins from the culture supernatant (S) were precipitated with 10% TCA (Sigma) at 4°C for 30 min. The pellet, recovered by centrifugation (13 000 g, 10 min), was washed with cold acetone and resuspended in TE buffer pH8 (Tris-HCl 10 mM EDTA 1 mM). Extraction of envelope proteins (E) of *C. glutamicum* was performed with SDS at high temperature. Briefly, the centrifuged cells were resuspended in Laemmli buffer and after heating 5 min at 100°C, cells were discarded after centrifugation. The supernatant containing the envelope proteins (E) and the proteins from the culture supernatant (S) were analyzed on SDS PAGE.

Extraction and analysis of the proteins from the capsule of *M. smegmatis* was performed as described by Sani et al. [22] using 1% Tween 80 and the proteins were precipitated with TCA before analysis on SDS PAGE.

2.7. Western blotting

Protein extracts (5 to 10 µg of total protein) were separated by SDS-PAGE (12%) and transferred to nitrocellulose membrane. Immunoblotting was performed using either anti-His6, anti-HA- or Concanavalin A peroxidase-conjugates (Roche) as recommended by the manufacturer. The blots were developed using clarity western ECL substrate (Bio-Rad). The ImageQuant LAS 500 was used for chemiluminescence detection.

2.8. Mass spectrometry analysis

-Sample preparation-

LppX SDS-PAGE bands, corresponding to different strains, were excised from the gel after Coomassie Blue staining. In-gel enzymatic digestion was performed using standard conditions. Briefly, protein bands were extensively washed with acetonitrile (ACN) and 100 mM ammonium carbonate. The excised bands were treated with 10 mM dithiothreitol (DTT) at 56 °C for 30 min. After DTT removal, cysteine carbamidomethylation was performed at room temperature for 30 min by addition of 55 mM iodoacetamide. After removal of the supernatant, the washing procedure was repeated, and gel slices were dried. Tryptic digestion was performed overnight at room temperature by addition of 20 µl of 10 ng/µl Porcine Gold Trypsin (Promega) diluted in 50 mM ammonium carbonate. Proteolytic peptides were extracted first by addition of 20 µl of 50% ACN and 0.1% formic acid and second by addition of 20 µl of 100% ACN. Tryptic peptides extracted were vacuum dried and resuspended in

212 0.05% ACN and 0.1% trifluoroacetic acid (TFA) prior to nano LC-MS/MS mass spectrometry
213 analysis.

214 **-Nano LC-MS/MS analysis-**

215 Nano LC-MS/MS analysis were performed with the triple-TOF 4600 mass spectrometer
216 (Absciex, Framingham, MA, USA) coupled to the Nano-RSLC system (Thermoscientific). Briefly,
217 peptides were desalted on a C18 reverse phase pre-column (C18 Acclaim Pepmap100, 3 μ m,
218 100 \AA , 75 μ m i.d., 2 cm length) using a loading buffer containing H₂O/ACN/TFA
219 (98%/2%/0.05%) at 5 μ l/min and were then eluted at a flow rate of 300 nl/min from the
220 reverse phase analytical C18 column (C18 Acclaim Pepmap100, 2 μ m, 100 \AA , 75 μ m i.d., 50 cm
221 length) using a 5-35% solvent B gradient for 40 min. Solvent B was 0.1% formic acid in 100%
222 ACN and solvent A was 0.1% formic acid in water. Nano LC-MS/MS experiments were
223 conducted in Data Dependent acquisition method by selecting the 20 most intensens
224 precursors for CID fragmentation with Q1 quadrupole set at low resolution for better
225 sensitivity and with a collision energy ramp (\pm 15%) set to 35 V.

226 **-Data analysis-**

227 Raw data were processed using MS data converter software (Absciex) and protein
228 identification was performed using the MASCOT search engine (Matrix science, London, UK)
229 against LppX sequences with carbamidomethylation of cysteines set as fixed modification.
230 Oxydation of methionines and glycosylation of serine and threonine were set as variable
231 modifications. Peptide and fragment tolerance were respectively set at 25 ppm and 0.05 Da.
232 Only peptides with mascot ions score higher than the identity threshold (30) at less than 1%
233 of false positive discovery rate are considered. For relative quantification of the LppX P₆₋₂₉

glycoforms, MS extracted ion chromatogram (XIC) were realized on LppX P₆₋₂₉ harboring glycosylation using peakview software 1.2 (Absciex).

2.9. Lipids extraction from culture supernatants, cell surface and cells of M. tuberculosis

Culture supernatants (50 ml) were sterilized by filtration, lyophilized and lipids were extracted following Bligh and Dyer protocol [23] and analyzed by LC/MS. Cells obtained from 100 ml cultures were extracted with 3 ml of water-saturated butanol for 16h. The butanol extracts were collected and filter sterilized. The remaining cell pellets were extracted with chloroform: methanol (1:2, v:v) once and chloroform: methanol (2:1, v:v) twice. The combined extracts were subjected to Folch wash, dried and analyzed by LC/MS. LC/MS was performed using an electrospray ionization/atmospheric pressure chemical ionization source in the positive mode on an Agilent 6220 time-of-flight mass spectrometer equipped with a MultiMode Source.

3. Results

3.1. Identification of the glycosylation sites of LppX-tb in *C. glutamicum*

In a previous study, we reported that LppX-tb was O-glycosylated in *C. glutamicum* [11]. The glycosylation sites were located within the N-terminal part of LppX-tb between amino acids 6 and 29 (Fig. 1). MS analysis identified heterogeneously glycosylated forms of LppX-tb carrying from 1 to 4 hexose units. In this study, we tried to identify the glycosylation site(s) within the peptide using conventional CID MS/MS techniques but unfortunately this was not successful. To determine the residues targeted by glycosylation in LppX-tb we thus performed a PCR-mediated approach to generate site-directed mutagenesis of putative glycosylation sites within the N-terminal part of LppX-tb. In this part of the polypeptidic chain, 3 hydroxyl-bearing amino acids were present (serine S16 and S20, threonine T18). All were predicted as putative glycosylation sites (Fig. 1) by the NetOGlyc 4.0 software [24]. Threonine T18 and serine S20 were individually substituted by an alanine leading to the mutated-genes LppX-tb T18A and LppX-tb S20A. As we failed in replacing serine S16 by alanine, this serine was deleted leading to the mutated-gene LppX-tb Δ S16. All mutated-genes were generated by PCR and cloned into the *C. glutamicum* expression vector pCGL482 under the control of the amyE promoter [11]. Recombinant strains were grown in BHI at 30°C overnight. After growth, cells were separated from the media by centrifugation and broken. Unbroken cells were removed and total membranes (inner and outer membranes) were recovered by high speed centrifugation. The membranes were solubilized in LDAO and solubilized membrane protein extracts were loaded on Ni-NTA column to purify the native and mutated His tagged LppX-tb. The purified proteins were analyzed by MS.

Different glycoforms of the N-terminal peptide were detected after proteolysis of the native LppX-tb protein (Fig. 2A): the peptide with no modification (P₆₋₂₉), the peptide with 1, 2, 3 and 4 hexose units (P₆₋₂₉ Hex1, P₆₋₂₉ Hex2, P₆₋₂₉ Hex3 and P₆₋₂₉ Hex4). The P₆₋₂₉ Hex2 form was the most abundant form detected (Fig. 2B). Analysis of the mutants shows no difference in the glycosylated pattern between the native LppX-tb and LppX-tb S20A. For the mutants LppX-tb ΔS16 and T18A, only P₆₋₂₉ and P₆₋₂₉ Hex2 were detected. A double-mutated LppX-tb gene ΔS16-T18A was finally constructed and did not reveal any glycosylated peptide (Fig. 2C). All these results demonstrate that S16 and T18 are both native glycosylation sites and that each site is diglycosylated in the native protein. It is interesting to note that the glycosylation of S16 and T18 was not depending on each other. Moreover, despite modification of the glycosylation pattern, LppX-tb variants ΔS16, T18A and ΔS16-T18A were still membrane-associated.

3.2. *Cg_PMT* is essential for glycosylation of LppX-tb in *C. glutamicum*

As seen previously, LppX-tb is O-glycosylated with hexoses but the exact chemical nature of the modification was not determined. Nevertheless alpha mannosidase deglycosylation experiment suggests that LppX-tb is indeed mannosylated in *C. glutamicum* [11]. A similar result is suggested in *M. tuberculosis* since LppX-tb was detected among the ConA affinity capture glycoproteome [6].

In *C. glutamicum*, different proteins were identified as glycosyltransferases. Among these proteins, Pmt (encoding gene NCgl0854) was identified as a protein O-mannosyl transferase which transfers mannose residues from lipid carriers to proteins [25]. To investigate the possible role of Pmt in the glycosylation process of LppX-tb, a mutant of *pmt* was constructed in *C. glutamicum*. The *pmt* mutant (*pmt::Km^r*) was generated by integration

of a kanamycin resistance cassette into the 5' part of the gene and the broad glycosylation protein pattern of the *pmt* mutant was tested. The wild type and *pmt::Km^r* *C. glutamicum* strains were grown in BHI. The cells and culture supernatant were separated by centrifugation. Treatment of whole cells with high concentrations of SDS is known to release cell wall components without affecting cell integrity in *Corynebacterium*. This method was used to extract proteins from the envelope. Proteins from the culture supernatant were recovered by TCA precipitation. Both proteins from the envelope and culture supernatant were transferred on SDS-PAGE and analyzed by immunoblotting with Concanavalin A peroxidase conjugate. As seen in Fig. 3A, several glycoproteins were detected both in the envelope and supernatant culture of the wild type strain. As expected no ConA reactive glycoproteins were detected in the *pmt::Km^r* strain. The *pmt* gene of *C. glutamicum* (*Cg-pmt*) was cloned in the expressing vector pVWEx2 under the control of the tac promoter and the recombinant plasmid was transferred in the *pmt* mutant for complementation studies. When the wild type *pmt* gene is expressed in *pmt::Km^r*, glycoproteins are again detected with a similar pattern to the wild type strain whether IPTG was added or not. As described in Mahne et al [25], our results confirm that Pmt is responsible for the glycosylation of secreted proteins in the culture supernatants. In addition, in this study we detected other *pmt*-dependent glycosylated proteins associated to the cell envelope of *C. glutamicum*.

As the *pmt::Km^r* mutant was confirmed, we specifically checked if glycosylation of LppX-tb was dependent of this gene by expressing LppX-tb in the mutant *pmt*. The LppX-tb gene was cloned in the expression vector pCGL482 and used to transform the wild type and *pmt::Km^r* mutant of *C. glutamicum*. LppX-tb was partially purified from the membranes of these strains and analyzed by SDS PAGE and western immunoblotting (Fig. 3B). As seen in Fig. 3B, a major protein of 25kDa was detected by Concanavalin A in the wild type strain. In the

mutant *pmt::Km^r*, a major band was detected slightly below than in the wild type strain and was not detected by the Concanavalin A conjugate suggesting that LppX-tb is not glycosylated in the *pmt::Km^r* mutant. To confirm that this is not related to a polar effect of the mutation, the *pmt* gene of *C. glutamicum* Cg-*pmt* cloned into the expression vector pVWEx2 was co expressed with LppX-tb in *pmt::Km^r*. In these conditions the migration behavior of LppX-tb was restored. The corresponding band was detected by Concanavalin A whether IPTG was added or not. Glycosylation of LppX-tb in the complemented strain was definitively confirmed by MS analysis (Suppl. Fig. S1). As for the wild type, different glycoforms of the peptide were detected (P₆₋₂₉ Hex2 and P₆₋₂₉ Hex4) as well as the 262 Da modification previously seen in LppX-tb by Mohiman et al. [11]. In the mutant *pmt::Km^r* MS analysis confirmed that the peptide P₆₋₂₉ was not glycosylated (Suppl. Fig. S1) which is in accordance with the absence of ConA binding seen on western immunoblot. These results demonstrate unambiguously that glycosylation of LppX-tb is dependent on Cg-*pmt* in *C. glutamicum*.

Complementation studies were also performed with the *M. smegmatis* *pmt* gene (Msmeg_5447). The PMT proteins encoded by Ms-*pmt* from *M. smegmatis* or Cg-*pmt* from *C. glutamicum* are quite similar with a sequence identity of 42% (Suppl Fig. S2). Moreover some residues important for the catalytic activity [25] are conserved in both Ms-*pmt* and Cg-*pmt*. Despite this similarity, the heterologous complementation of the *C. glutamicum* *pmt::Km^r* mutant with Ms-*pmt* was unsuccessful. Neither the glycoproteins from the envelope and supernatant culture nor LppX-tb were detected by Concanavalin A in the complemented strain (Fig 3C and 3D). The absence of glycosylation of LppX-tb was also confirmed by MS analysis. As the absence of glycosylation might be related to an expression problem, we tried to purify the protein PMT expressed from Ms-*pmt* during heterologous expression. The same experiment was performed with Cg-*pmt* as a positive control. In both cases, we were not able

to detect the PMT proteins. Although this result suggests that very low amounts of Cg-PMT, is sufficient to allow complementation, it does not allow us to conclude for the absence of complementation by *Ms-pmt*.

3.3. Glycosylation of LppX-tb in *M. smegmatis*

Here, before proceeding on the role of LppX glycosylation, it was important to check whether LppX-tb glycosylation sites and pattern in *Mycobacteria* are the same as those found heterogously in *C. glutamicum*. For this, we choose to work in *M. smegmatis*, a fast growing species which is easily manipulated and in which several glycosylation studies have been performed so far.

M. smegmatis strain MC²155 carrying pMV261-LppX-tb [8] was grown in BHI and LppX-tb was purified as described in materials and methods. A similar experiment was performed in parallel with *C. glutamicum* using pCgl482-LppX-tb. Both purified proteins were analyzed by western immunoblot using Concanavalin A peroxidase conjugate. As expected, LppX-tb was detected by the ConA conjugate when purified from *C. glutamicum*. Surprisingly, we were not able to visualize any signal when LppX-tb was purified from *M. smegmatis* suggesting that either LppX-tb is not mannosylated or that the glycosylation level of LppX-tb is not high enough to allow detection. In order to discriminate between these hypothesis, LppX-tb was analyzed by MS analysis. As shown in Fig. 4, LppX-tb purified from *M. smegmatis* was found to be glycosylated on the N-terminal part of the protein. Several forms of peptide P₆₋₂₉ were detected. The non-glycosylated form was the most abundant but small amounts of the Hex1 and Hex2 glycoforms were also detected. This low amount of glycoforms detected in *M. smegmatis* may account for the absence of detection by Concanavalin A.

As LppX-tb was found glycosylated in *M. smegmatis*, we wanted to determine if the glycosylation sites of LppX-tb in *M. smegmatis* were the same as in *C. glutamicum*. Mutations of the putative glycosylation sites S16, T18 and S20 of LppX-tb were generated according to the same procedure as for *C. glutamicum* except that the mutated genes were cloned in pMV261 an expression vector for mycobacteria. The recombinant plasmids were introduced into *M. smegmatis* MC²155 and the strains were grown in Middlebrook 7H9 broth supplemented with Middlebrook ADC enrichment and 0.5% glycerol and Tween 80 (0.05 %). The different LppX-tb proteins were purified from the membrane fraction as described in materials and methods and analyzed by MS (Fig. 5). When expressed in *M. smegmatis*, different glycoforms of the peptide (peptide with no modification P₆₋₂₉, peptide with 1 and 2 hexose units respectively P₆₋₂₉ Hex1 and P₆₋₂₉ Hex2) were detected with native LppX-tb as well as with the variant LppX-tb S20A. For the variant LppX-tb ΔS16 and T18A, only P₆₋₂₉ and P₆₋₂₉ Hex1 were detected demonstrating that both S16 and T18 are glycosylation sites and that each site can be monoglycosylated. This was confirmed by analysis of the double mutant LppX-tb ΔS16 T18A in which no glycosylated peptide could be found. As for *C. glutamicum* it is interesting to note that the glycosylation of S16 and T18 was not depending on each other. These results show that the residues targeted by glycosylation in LppX-tb are identical in both *C. glutamicum* and *M. smegmatis*. This result is in line with a recent work supporting similar process of glycosylation in *M. smegmatis* and *M. tuberculosis* [26].

3.4. Native and ΔS16 T18A variant of LppX-tb are associated to the outerlayer of *M. smegmatis*

In our work, when expressed in *M. smegmatis*, LppX-tb was detected in the membrane fraction. In *M. tuberculosis* and *M. bovis* LppX-tb and its ortholog were found associated to the outermost layers of the cells that is the cell wall [27–29] and the cell surface [27]. In these

mycobacteria, the cell wall is surrounded by a capsular layer [22]). We decided to analyze the mycobacterial capsular layer of *M. smegmatis* to see if LppX could also reach this outerlayer and whether S16 and T18 glycosylation was necessary for this localization.

The mycobacterial capsular layers of the different *M. smegmatis* strains MC²155 carrying pMV261, pMV261-LppX-tb and pMV261 LppX-tb Δ S16 T18A were extracted from the cells as described by Sani et al. [22]. For this experiment Tween 80 was not added in the culture media since this detergent is known to shed the capsule into the medium. After extraction, the proteins of the mycobacterial capsular layer were precipitated with TCA and analyzed by SDS PAGE and immunoblotting (Fig. 6A). The non-recombinant strain was used as a negative control. As seen in Fig. 6A similar amounts of proteins were extracted from the different strains. According to the immunoblot, LppX was only detected in the outerlayer of both recombinant strains whether S16 and T18 residues of LppX were modified or not.

As we noticed minor differences on SDS PAGE patterns of the different strains, an additional capsular extraction was performed and the protein content of the mycobacterial capsular layers were further analyzed on 8% SDS-PAGE (Fig. 6B). As seen in Fig. 6B, an additional protein was specifically detected in the strain expressing the Δ S16 T18A variant of LppX-tb. This protein was identified by MS as the 60-kDa chaperonin GroEL1 from *M. smegmatis* (Suppl Fig. S3). Interestingly, the presence of GroEL1 in the capsule layer of *M. smegmatis* and *M. tuberculosis* was previously reported [22]. Here we show that this protein is specifically enriched in the capsule of *M. smegmatis* expressing the LppX-tb Δ S16 T18A variant and this may suggest a specific function related to the absence of LppX glycosylation.

3.5. The Δ S16 T18A variant of LppX-tb is still able to promote translocation of phthiocerol dimycocerosates to the surface of M. tuberculosis

In *M. tuberculosis* phthiocerol dimycocerosates are required for virulence of the bacteria [7]. Since LppX-tb is necessary for translocation of these molecules to the surface of *M. tuberculosis* and, because mannosylation was shown to contribute to *M. tuberculosis* virulence, we reasoned that glycosylation of LppX-tb might be important for LppX-tb function. As disruption of LppX-tb in *M. tuberculosis* had no effect for growth in 7H9 broth [7], this mutant was chosen for complementation studies with the native and the double mutated $\Delta S_{16} T_{18}A$ LppX-tb gene. As previously described, in contrast to *M. tuberculosis* wild type strain, the LppX-tb null mutant fails to release DIM into the culture medium but retains the ability to translocate these lipids across the plasma membrane [7]. We therefore decided to check the repartition of DIM in various mutants: the LppX-tb knock-out strain and the knock-out strains complemented with native or mutated LppX-tb cloned in the expression plasmid pVV16 (Fig. 7). For this experiment cells were grown in 7H9 medium containing ADC and 0.05% Tween 80 allowing the release of some outermost components of the cells in the culture media. In these conditions DIM molecules were present in the cells of all strains and all strains produced similar amounts of DIM (Fig. 7A). The chromatography profile of the DIM molecules was however slightly different in the LppX-tb knock-out cells as compared to the other cells. As expected and seen previously [7] DIM molecules were absent from the culture filtrates of the knock-out strain. However DIM molecules were present in the culture filtrates of all complemented strains including the strain harboring the double mutation $\Delta S_{16} T_{18}A$ (Fig. 7A). This result demonstrates that the presence of DIM in the culture filtrate of *M. tuberculosis* is not dependent on S16 and T18 LppX glycosylation of LppX-tb and therefore neither the localization of DIM in the outerlayer of *M. tuberculosis*.

Further analysis of the composition of DIM was performed by LC/MS. As shown in Fig. 7B, cells of the knock-out strain mainly contain DIM A molecules whereas cells (Suppl. Fig. S4)

432 and culture filtrates of the two complemented mutants mainly contain DIM B molecules (Fig.
433 7B). Again the S16 and T18 LppX glycosylation state of LppX-tb has no impact in the
434 composition of DIM molecules.

4. Discussion

In this article we used heterologous hosts to determine the glycosylation sites of the *M. tuberculosis* protein LppX-tb. A non glycosylated LppX-tb variant was then constructed and used to study the impact of glycosylation on LppX-tb function in *M. tuberculosis*.

4.1. The glycosylation sites S16 and T18 are localized in the unfolded N-terminal part of LppX-tb

No specific target sequon is known for *O*-glycosylation in contrast with *N*-glycosylation. However *O*-glycosylation sites are most often localized within regions of low sequence complexity [1]. Here in agreement with these studies we showed that LppX-tb glycosylation sites are localized in the N-terminal region of the lipoprotein LppX-tb in a region lacking any secondary structure [7]. Moreover as seen for other mannosylated mycobacterial proteins [5,30] these modified sites are within a region enriched with amino acids proline and alanine.

Both S16 and T18 residues are also present in the corresponding LppX-tb ortholog of *M. bovis* suggesting potential glycosylation at the same residues in this protein. In the *M. leprae*, *M. marinum* *M. ulcerans* and *M. goodii* LppX-tb-orthologs these residues differ at position 16 and/or 18 but their N terminal regions are enriched with threonine and serine residues with some of them predicted as potential glycosylation target (Suppl. Fig. S5).

4.2. The glycosylation sites S16 and T18 of LppX-tb are identical in *C. glutamicum* and *M. smegmatis* but the glycosylated forms detected are different.

Whether LppX-tb is expressed in *C. glutamicum* or *M. smegmatis*, the lipoprotein is found to be modified by a hexose (probably mannose) at exactly the same glycosylation sites. However the glycosylation pattern differs between both bacteria. In *M. smegmatis* up to 2

hexoses are added to LppX-tb. The same result was reported during expression of LppX-tb in other mycobacterium species *M. bovis* BCG [9] and *M. abscessus* [10]. In *C. glutamicum* up to 4 hexose residues were found associated to LppX-tb during expression together with an unknown modification of 262 Da occasionally detected in the N-terminal peptide of LppX-tb as seen previously [11]. This modification was never detected when LppX-tb was expressed in *M. smegmatis*.

These results point out homologous sequence recognition in mycobacteria and corynebacteria but also minor differences reflecting their specific metabolism. In mycobacteria the highly glycosylated form of LppX-tb (Hex 3 and Hex 4) and the 262 Da modification detected in *C. glutamicum* may either be absent and/or the amounts synthesized not high enough to allow detection.

4.3. Glycosylation of LppX-tb is performed by Pmt

In *C. glutamicum* the gene *pmt* (Cg1014 or NCgl0854) encoding for a mannosyltransferase responsible for protein-mannosylation was previously identified [25]. When expressed in the interrupted *pmt* mutant of *C. glutamicum*, LppX-tb is no longer glycosylated. Glycosylation is recovered when complementation with the Cg-pmt gene is performed. Thus our results confirm that Cg-pmt is involved in the glycosylation process of LppX-tb in addition to the polyprenol phosphate mannose synthase (cg-ppm1) and the apolipoprotein N-acyl transferase (cg-ppm2 or Int) in *C. glutamicum* [11]. Moreover as previously proposed [11] mannosylation of LppX-tb is confirmed. The exact role of Cg-pmt remains to be elucidated since Cg-pmt could be responsible both for the initial attachment of the mannosyl residue to the target residues and the elongation of the oligosaccharide chain.

479 Alternatively other(s) glycosyltransferase(s) might be needed for the elongation step.
480 Previously identified glycosyltransferases in *C. glutamicum* [31] could be involved.

481 No complementation was noticed during heterologous expression of Ms-pmt of *M.*
482 *smegmatis* in the interrupted pmt mutant of *C. glutamicum*. This result was surprising since
483 we already observed successful complementation of *C. glutamicum* mutant with a *M.*
484 *smegmatis* gene [12]. However different hypothesis might explain this result. First during the
485 mannosylation process Pmt transfers mannose from polyprenol phosphate mannose (PPM)
486 to a target protein during its translocation across the membrane. In *M. tuberculosis* this
487 process requires interaction with the Sec complex [32]. Thus the interaction of Ms-pmt with
488 the Sec translocon of *C. glutamicum* might not be functional. Second the polyprenols involved
489 in mannose translocation in actinomycetales differ in chain length and saturation of the
490 isoprene unit [5]. Only C55-polyprenolmannose was detected in *C. glutamicum* [33] whereas
491 C35, C40 and C50-PPM were found in *M. smegmatis* [34]. The protein Ms-pmt may not be able
492 to recognize the C55-PPM of *C. glutamicum* and/or transfer the mannose. Finally the Ms-pmt
493 may also be not correctly expressed or localized in the cell.

494 According to our results (that is evidence of mannosylation of LppX-tb in different
495 actinobacteria and the role of Cg-Pmt) and considering that the proteins Pmt from *C.*
496 *glutamicum*, *M. smegmatis* et *M. tuberculosis* are homologous [4,25] Sm-Pmt and Mt-Pmt
497 are certainly responsible for LppX-tb mannosylation in the respective bacteria. Moreover as
498 the glycosylated residues of LppX-tb are the same in *C. glutamicum* and *M. smegmatis* we can
499 conclude that Cg-Pmt and Sm-Pmt are able to recognize identical glycosylation pattern in
500 LppX-tb. Although very likely, more experimental results are needed to expand this conclusion
501 to a larger group of proteins.

4.4. Distribution of DIM in the mycobacterial cell is not altered whether S16 and T18 residues of LppX-tb are glycosylated or not

In lipoproteins, an intrinsically disordered domain is generally found between the N terminal lipid anchor and the functional domain of the protein [35]. Various functions are associated with intrinsically disordered proteins or regions such as connecting ordered domains, regulation, molecular recognition and signaling and assisting in protein folding [3]. In lipoproteins this unfolded domain contains the lipoprotein sorting signal and is thought to properly position the lipoprotein [35]. Moreover Prates et al. [3] proposed that the elongation effect due to glycosylation of unfolded domains may be important to provide the optimum distance between the connected domains for protein function. As the LppX-tb glycosylated residues were positioned in this part of the lipoprotein, glycosylation could act as a signal for its export and/or function. These data prompted us to evaluate the possible impact of LppX-tb glycosylation on its localization and function. Our results show that the outerlayer localization of WT and modified LppX-tb Δ S16T18A in *M. smegmatis* is identical. Moreover LppX-tb harboring mutated S16 and T18 residues is still able to promote DIM translocation across the cell envelope in *M. tuberculosis* suggesting that its function is not significantly impaired. A similar result was also described for LprG, a structurally homolog protein of LppX-tb. Indeed, LprG, which is O-mannosylated with a unique hexose on a threonine residue [26] was also shown to be essential for correct surface localisation of complex lipids (mannose-capped lipoarabinomannan (LAM)) in *M. tuberculosis* [36]. Interestingly, Alonso et al. showed that the surface localization of LAM in a *pmt* mutant of *M. tuberculosis* is not impaired [26]. Thus it seems that the distribution of complex lipids such as LAM and DIM in the mycobacterial cell is not dependent on the glycosylation state of their protein carriers LprG and LppX-tb

respectively. Consequently these results suggest that the absence of LppX and LprG glycosylation does not account for the attenuation of virulence observed in the pmt mutant.

4.5. Is there a role for GroEL1 in DIM translocation?

In absence of mannosylation the function of LppX is not affected which is quite surprising as generally glycosylation is thought to affect protein folding and stability. However one can imagine that in the absence of glycosylation other factors may substitute for assisting LppX stability to maintain a sufficient level of DIM translocation at the cell surface. In that respect it is interesting to notice that Wang et al [37] previously discovered that the presence of phthiocerol dimycocerosates in *M. bovis* BCG is dependent on GroEL1. Because we show that the amount of GroEL1 is largely increased in the capsular layer of *M. smegmatis* only when the non-glycosylated variant of LppX-tb is expressed, it is thus tempting to propose that GroEL1 may be involved in the DIM translocation through an important role on LppX folding and/or stability. Indeed in the absence of glycosylation, the N-terminal non ordered chain of LppX may not be correctly extended and this effect could be reversed by GroEL1 overexpression. By extension, in *M. tuberculosis*, overexpression of GroEL1 could also counteract the absence of glycosylation of LppX-tb Δ S16-T18A and explain that DIM are still translocated at the cell surface. Further experiments are needed to validate this attracting hypothesis.

543 **Conflict of interest**

544 None declared

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666 **Legends to figures**

667 **Fig. 1. Amino acid sequence of the mature lipoprotein LppX.** Bold letters indicate the
668 glycosylated peptide P₆₋₂₉ identified by Mohiman et al. [11]. The HA and His₆ epitopes are
669 highlighted. The underlined amino acids were predicted as putative glycosylated site
670 (prediction with NetOGlyc 4.0 Server <http://www.cbs.dtu.dk> [24]).

671 **Fig. 2. (A)** Extracted ion chromatograms and **(B)** relative abundances of the LppX P₆₋₂₉
672 glycoforms observed during expression of wild type WT and mutated lppX Δ S16, -T18A and -
673 S20A genes in *C. glutamicum* (CG). **(C)** Extracted ion chromatogram of LppX P₆₋₂₉ glycoforms
674 observed during expression of the mutated lppX Δ S16 T18A genes in *C. glutamicum*. All strains
675 were grown in BHI. The different glycoforms detected are the peptide with no modification
676 (P₆₋₂₉), the peptide with 1, 2, 3 and 4 hexose units (respectively P₆₋₂₉ Hex1, P₆₋₂₉ Hex2, P₆₋₂₉
677 Hex3 and P₆₋₂₉ Hex4). The amino acid sequence of the wild type and mutated 6 – 29 peptides
678 are mentioned in the right upper part of the figures using a red letter for the substituted amino
679 acid and an asterisk for the deleted one.

680 **Fig. 3. Pmt dependent protein glycosylation in *C. glutamicum*.**

681 Western blot analysis of glycoproteins detected in the envelope (E) or in the culture
682 supernatant (S) of the wild type 13032 strain, *pmt::Km^r mutant* and *pmt::Km^r mutant*
683 complemented by *Cg-pmt* **(A)** or *Ms-pmt* **(C)**. Glycoproteins were detected using Concanavalin
684 A peroxidase conjugate. Partial purification of LppX from the wild type strain 13032, *pmt::Km^r*
685 mutant and *pmt::Km^r mutant* complemented by *Cg-pmt* **(B)** or *Ms-pmt* **(D)**. (Left) Protein
686 pattern on 12% SDS PAGE after Coomassie blue staining. (Right) Glycoprotein pattern after
687 Western immunoblot using Concanavalin A peroxidase conjugate.

688 For complementation studies cells were grown in presence (+) or absence (-) of IPTG.

Fig. 4. (A) Relative abundance of the LppX P₆₋₂₉ glycoforms observed during expression of the wild type *lppX* gene (WT) in *C. glutamicum* (CG) and *M. smegmatis* (MS) grown in BHI. **(B)** Extracted ion chromatogram of the LppX P₆₋₂₉ glycoforms detected in *M. smegmatis* (MS). The different glycoforms detected are the peptide with no modification (P₆₋₂₉), the peptide with 1, 2, 3 and 4 hexose units (P₆₋₂₉ Hex1, P₆₋₂₉ Hex2, P₆₋₂₉ Hex3 and P₆₋₂₉ Hex4)

Fig. 5. Relative abundances of the LppX P₆₋₂₉ glycoforms observed during expression of WT and mutated *lppX* Δ S16, -T18A, -S20A and Δ S16-T18A genes in *M. smegmatis* (MS) grown in Middlebrook 7H9 broth supplemented with ADC, glycerol and Tween. The different glycoforms detected are the peptide with no modification (P₆₋₂₉), the peptide with 1 and 2 hexose units (P₆₋₂₉ Hex1 and P₆₋₂₉ Hex2).

Fig. 6. LppX is found in the outermost layer of *M. smegmatis*. *M. smegmatis* Mc²155 carrying pMV261, pMV261-LppX and pMV261-LppX Δ S₁₆ T₁₈A were grown in Middlebrook 7H9 broth supplemented with 0.5% glycerol. Mycobacterial capsule proteins were extracted with Tween as described by Sani et al. [22], separated on a 12% SDS-PAGE **(A)** stained with coomassie blue (Left) and analyzed by western-immunoblot using anti HA peroxidase conjugate (Right). Additional and refined analysis was performed on a 8% SDS-PAGE stained with coomassie blue **(B)**. The additional protein identified by MS is indicated (★).

Fig. 7. LC-MS analysis of the distribution of DIM in the cells (cells) and culture filtrates (Culture filtrate). **(A)** Extracted ion chromatograms of DIM molecules from the *lppX* knock-out strain (KO) and the knock-out strains complemented with wild-type (WT) or mutated (Δ S16-T18) *lppX*. DIM molecules are absent from the culture filtrate of the knock-out strain, but present in the culture filtrates of both complemented mutant strains. **(B)** Mass spectra of DIM molecules extracted from the cells of the knock-out mutant, and from the culture filtrates

712 of the mutant complemented with WT or mutated *lppX*. Note that the cells of the knock-out
713 strain contain mainly DIM A molecules whereas cells (Suppl. Fig. S4) and culture filtrates of
714 the two complemented mutants mainly contain DIM B molecules. DIM A molecules are
715 derivatives of DIM B molecules in which a keto group is replaced with a methoxy group at the
716 terminus of the molecules. The ^{12}C isotope peaks characteristic of DIM A and DIM B molecules
717 are labeled with their observed m/z $[\text{M}+\text{NH}_4]^+$ values and their respective number of carbon
718 atoms.

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723 **Tables**

724 **Table 1.** Primers used in this study for the cloning and disruption of *pmt* and obtention of the
 725 mutated *lppX* genes. The mutagenic and flanking primers used to generate site directed
 726 mutations in the *lppX* gene are indicated. The mutated *lppX* genes were cloned into the
 727 expression vector pCGL482 and pMV261.

Gene or plasmid	Primers
Cg- <i>pmt</i> cloning	DirCgPmt- <i>Xba</i> I 5'- CGCTCTAGAGCATCTAGCTTCCCACAAATGC-3' RevCgPmt6His- <i>Bgl</i> II 5'-CGCAGATCTTTAATGATGATGATGATGATGGCGCCA GCTTGGAAC-3'
Ms- <i>pmt</i> cloning	DirMsmPmt- <i>Xba</i> I 5'- GCGTCTAGAGCCGAGTGTCACGACCGACAGC-3' RevMsmPmt6His- <i>Bam</i> HI 5'-CGCGGATCCTTAGTGATGGTGATGGTGATGGCG CCAGCTCGGCAAC-3'
<i>pmt</i> disruption	PMT1-1 5'-GCCAAAGTTCAAGTGG-3' PMT1-3 5'-TTACAGGTTTGAGCAGTGC-3' PMT1-4 5'-ATACACCTGTGGTGAAAC-3' PMT1-6 5'-AAGAGCTGCAGGATGGAGTC-3' M13R 5'- CAGGAAACAGCTATGAC-3' M13F(-20) 5'- GTAAAACGACGGCCAG-3'
Mutated <i>lppX</i> gene :	Mutagenic primers
<i>lppX</i> Δ S ₁₆	Rev LppX S16delta 5'-GGGTCGGACGCCGTCGGCACGGGAACACCCTGTTCC-3' Dir LppX S16delta 5'-GGAACAGGGTGTTCCCGTGCCGACGGCGTCCGACCC-3'
<i>lppX</i> T ₁₈ A	Rev LppX T18A 5'-CGGGGTCGGACGCCGcCGGGCTCACGGG-3' Dir LppX T18A 5'-CCCGTGAGCCCGGCGGCGTCCGACCCCG-3'
<i>lppX</i> S ₂₀ A	Rev lppx-S20A 5'- CACTCGGGTGCCGCCGGCTGGGGCGCGAGGAGC-3' Dir LppX S20A 5'- GTGAGCCCGACGGCGGCCGACCCCGCGCTCCTCG-3'
<i>lppX</i> Δ S ₁₆ -T ₁₈ A	RevS16deltaT18A 5'-CGGGGTCGGACGCCGcCGGCACGGGAACACC-3' Dir S16 deltaT18A 5'-GGTGTTCCTGTCGGCGCGGCGTCCGACCCCG-3'
Cloning plasmid	Flanking primers
pMV261	Dir pMV261 seq 5'- GGAATCACTTCGCAATGGCC- 3'

Rev LppX *Eco*RI 5'-CCACCACCATCACTAGAATTCGAGC-3'

pCGL482

482-S2 : 5'- GCAGAATAAATGATCCGTCG-3'

482-AS: 5'- GATATGATCATTATTCTGC-3'

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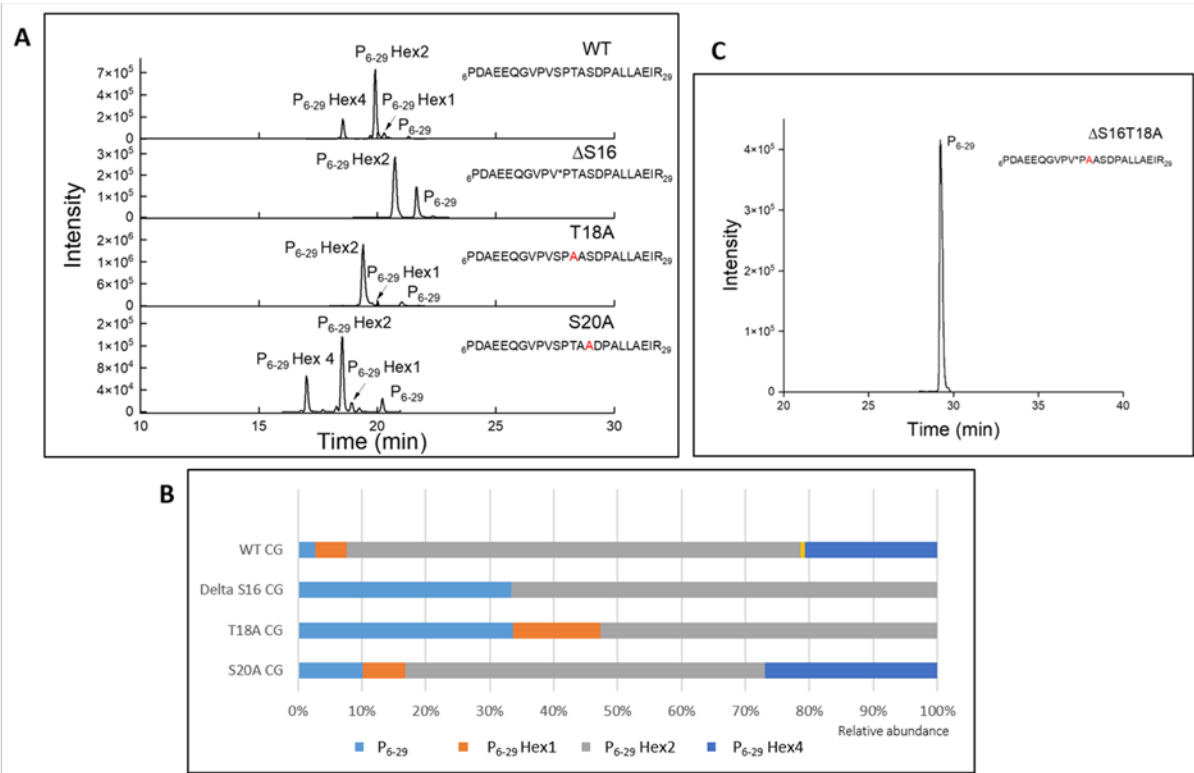
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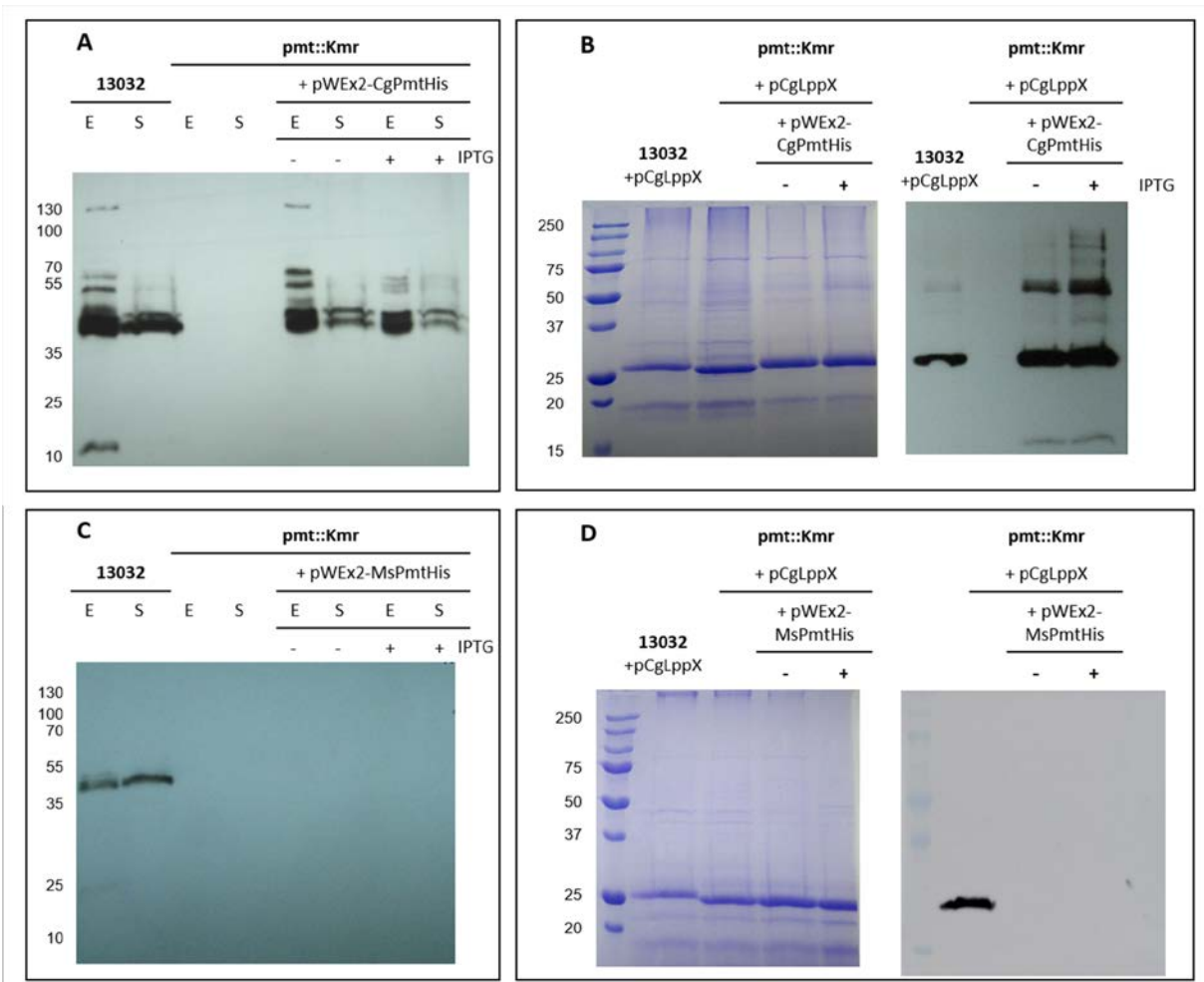
730 Fig. 1

CSSPKPDAEEQGVPVSPTASDPALLAEIRQSLDATKGLTSVHVAVRTTGKVDSLGITS
ADVDVRANPLAAKGVCTYNDEQGVFPRVQGDNISVKLFDDWSNLGSISELSTSRVL
DPAAGVTQLLSGVTNLQAQGTEVIDGISTTKITGTIPASSVKMLDPGAKSARPATVW
IAQDGSHHLVRASIDLGSGSIQLTQSKWNEPVNVDYPYDVPDYATWHHHHHHH

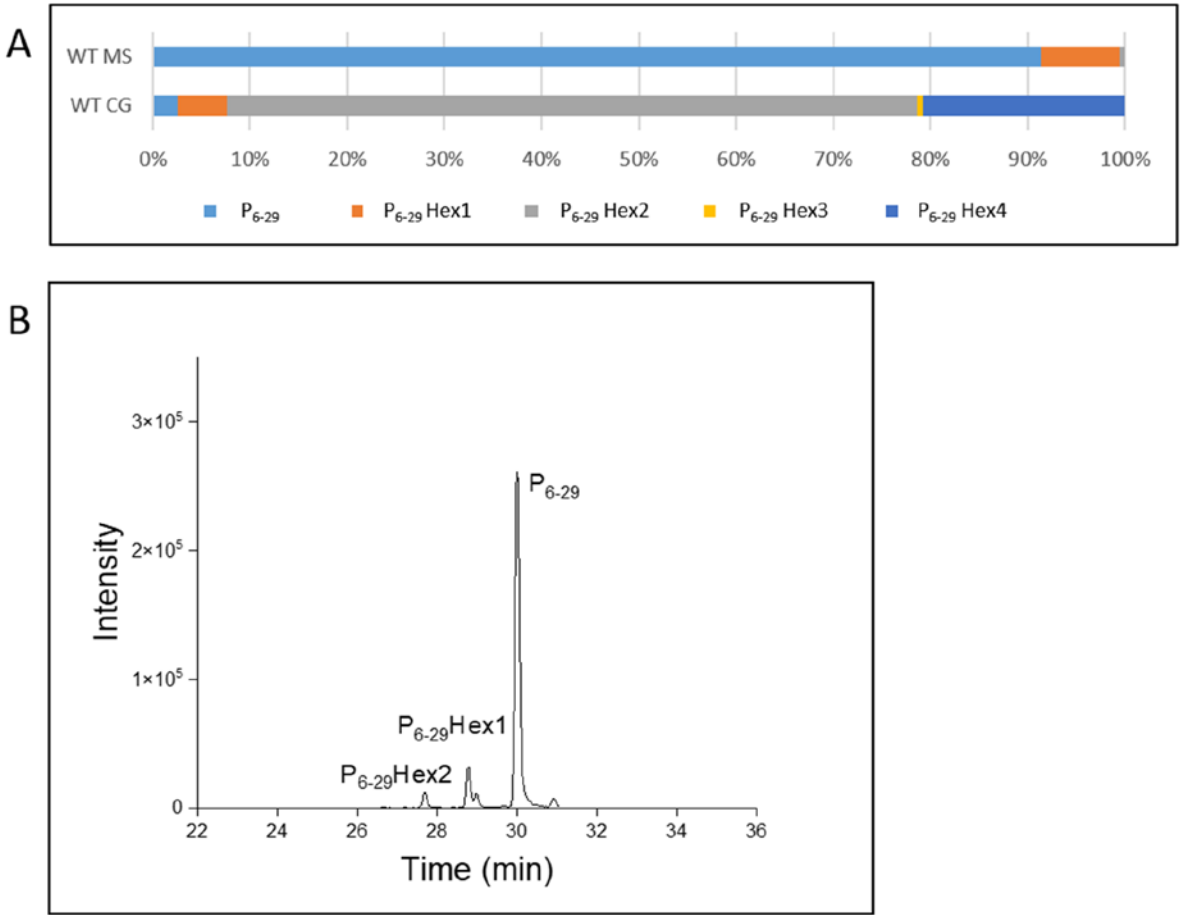
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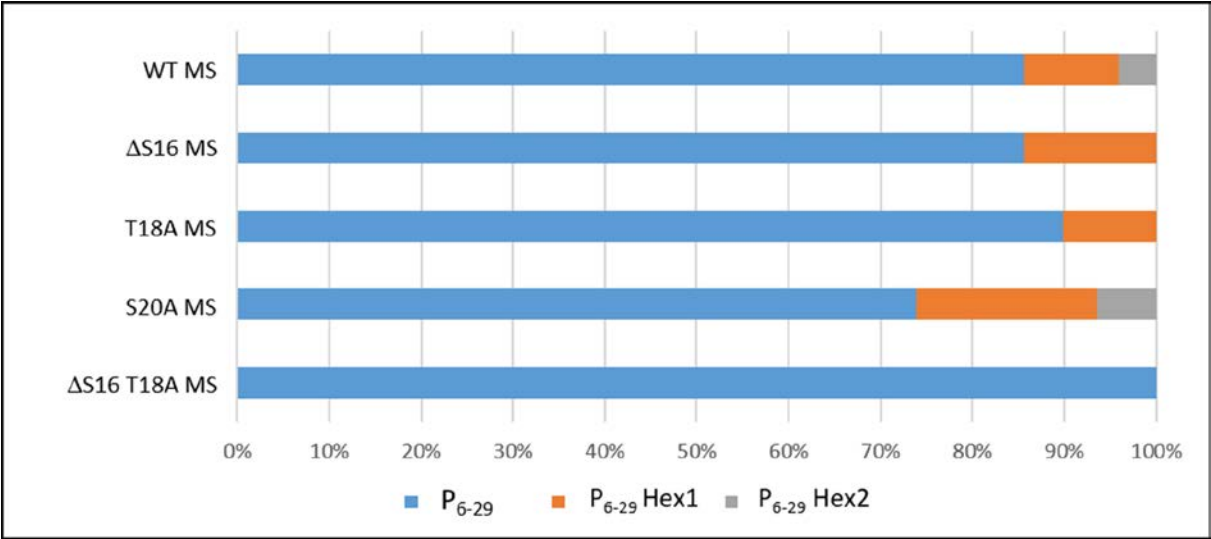
739 Fig.4



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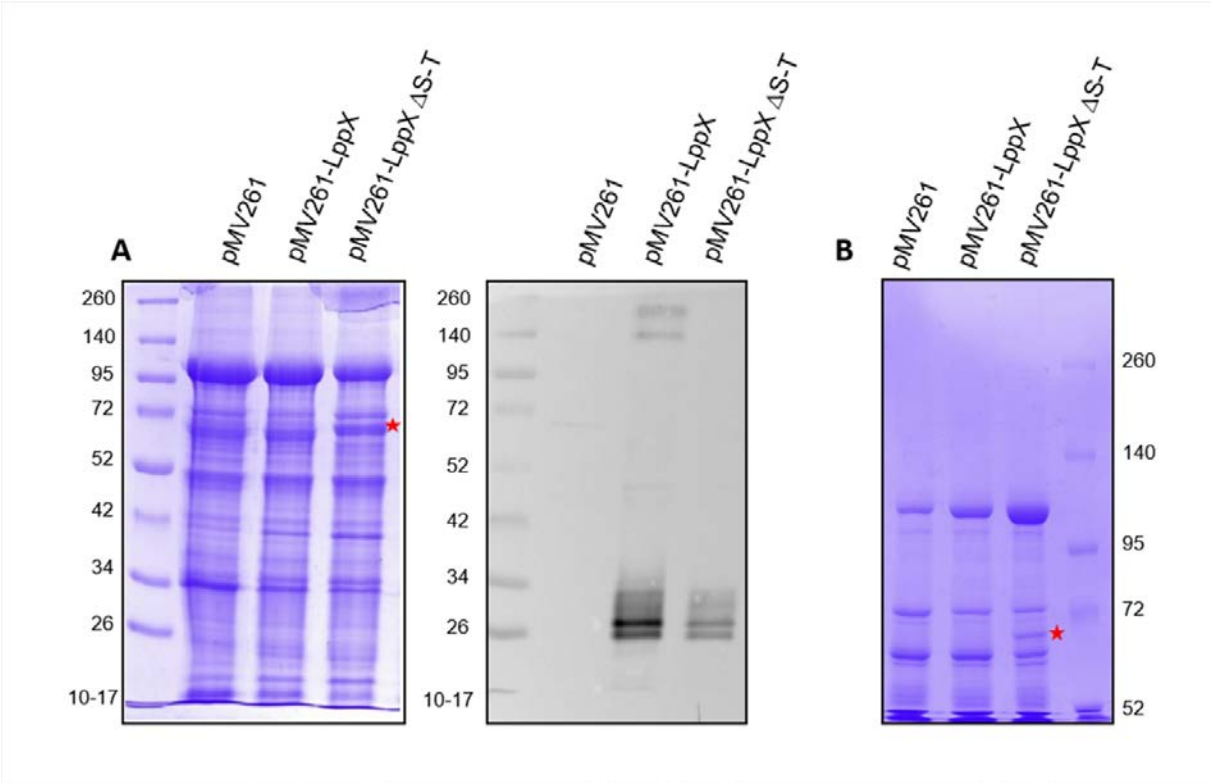
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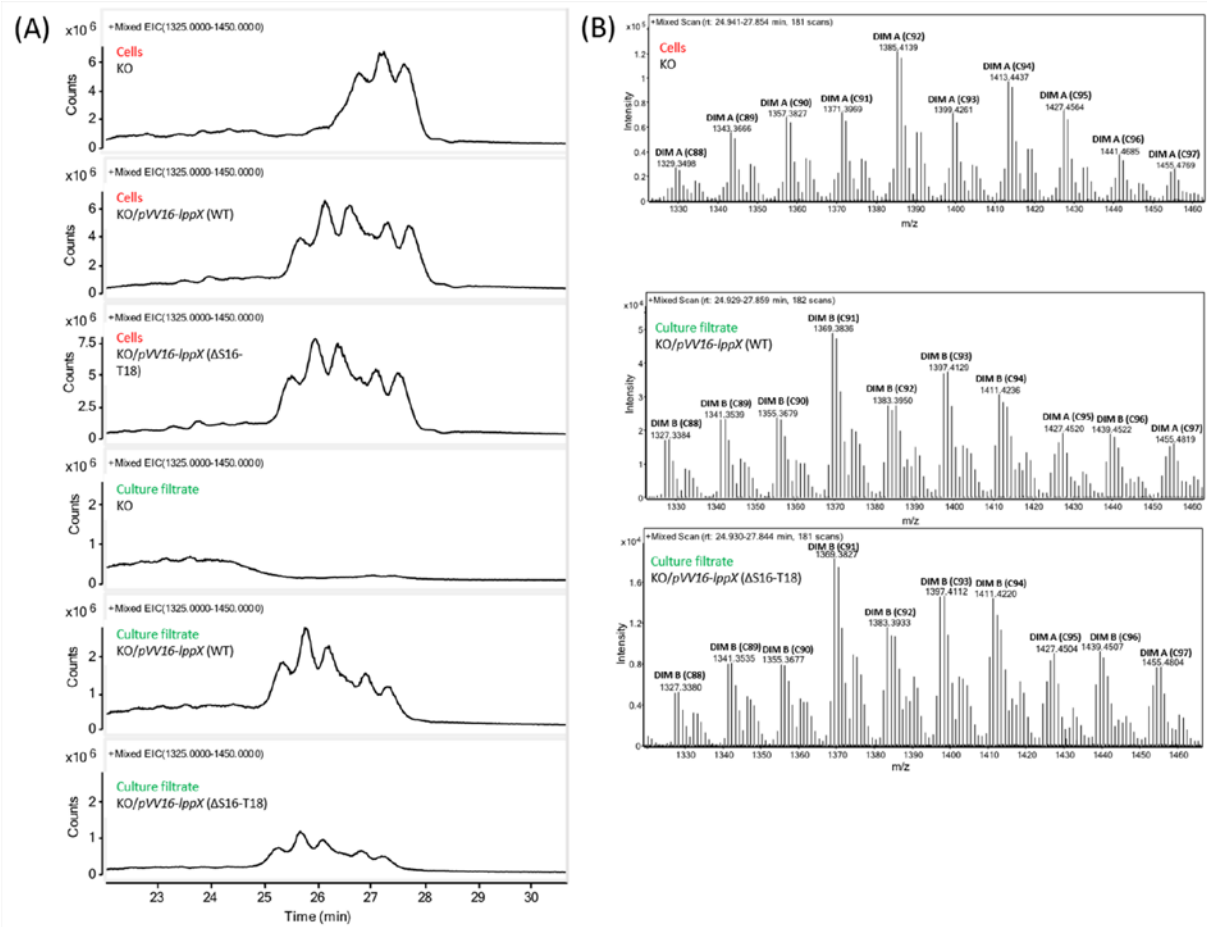
742 Fig. 5



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Supplementary material

Suppl Fig. S1. Complementation of the *pmt::Km^r* mutant of *C. glutamicum* with *Cg-pmt*.

(A) Mass spectrum of the LppX₆₋₂₉ peptide and (B) relative abundances of the LppX P₆₋₂₉ glycoforms observed during expression of *lppX*-tb in the *pmt::Km^r* mutant complemented by *Cg-pmt* of *C. glutamicum*. For the complementation studies cells were grown in presence (+) or absence (-) of IPTG. (C) Extracted ion chromatograms of the LppX P₆₋₂₉ glycoforms observed during expression of *lppX*-tb in the *C. glutamicum* wild-type strain (WT) and *pmt::Km^r* mutant.

Suppl Fig. S2. Sequence alignment of the PMT protein from *C. glutamicum* (PMT_Coryneglu) and *M. smegmatis* (PMT_Mycosmeg) using EMBOSS Needle <https://www.ebi.ac.uk> [38] (Identity : 228/539 (42.3%); Similarity : 310/539 (57.5%); Gaps : 42/539 (7.8%)). The conserved residues seen in various PMT proteins [25] are framed in blue.

Suppl Fig. S3. Mass spectrometry identification of the additional protein found in the outerlayer of *M. smegmatis* MC²155 expressing the modified LppX-tb LppX ΔS₁₆ T₁₈A.

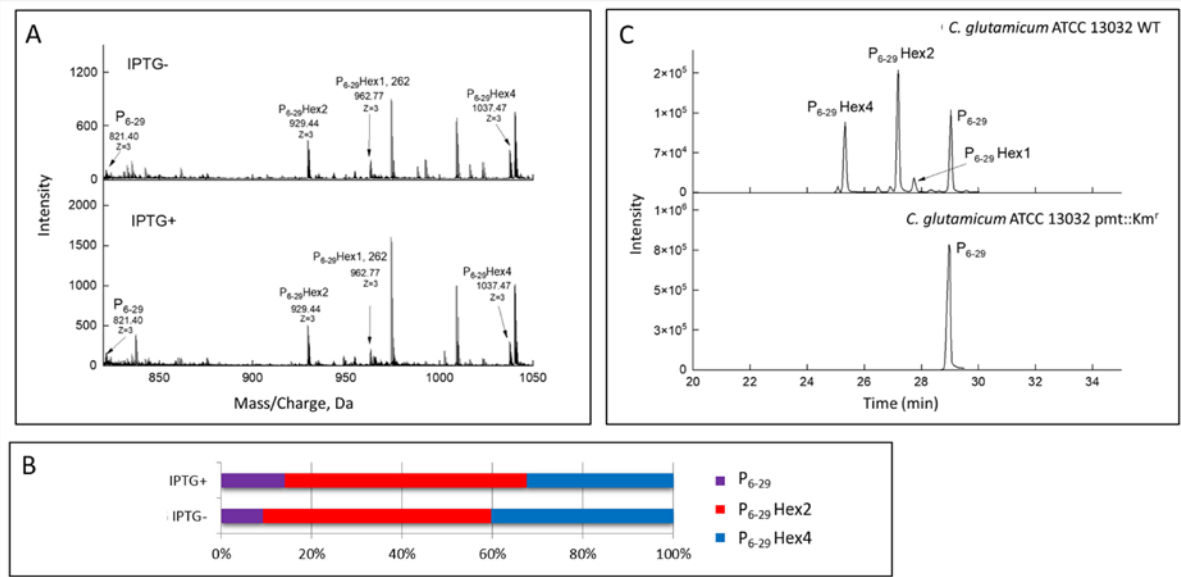
Identified peptides (A) and sequence coverage (B) of the 60kDa Chaperonin 1 (OS=*Mycobacterium smegmatis* (strain ATCC 700084 / mc(2)155) OX=246196 GN=groL1 PE=1 SV=1) resulting from in-gel trypsin digestion and Peptide-mass fingerprint (PMF) analysis.

Suppl. Fig. S4. Mass spectra of DIM molecules extracted from the cells of the *lppX* knock-out mutant (KO) complemented with WT or mutated (ΔS16-T18) *lppX*. The ¹²C isotope peaks characteristic of DIM A and DIM B molecules are labeled with their observed *m/z* [M+NH₄]⁺ values and their respective number of carbon atoms.

Suppl. Fig. S5. N- terminal predicted amino acid sequences of the mature LppX-tb orthologs of *Mycobacterium bovis*, *Mycobacterium leprae*, *Mycobacterium marinum*, *Mycobacterium ulcerans* and *Mycobacterium goodnae*. The predicted O-glycosylation sites are indicated with

774 bold letters (prediction with NetOGlyc 4.0 Server <http://www.cbs.dtu.dk> [24] and the position
775 of the glycosylated amino acids 16 and 18 in LppX-tb are framed in blue.

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PMT_Coryneglu	1	-----MSQALPVRDQGRDQGIFAGTLPPAPPKFKWTRLD-TYTW-	38
PMT_Mycosmeg	1	MTALDPTDPTAGRSAPL-----ISPGVIP-PPDF--GPLDRAQGWA	39
PMT_Coryneglu	39	--AIIAVFALVTRFTGLSSATASGTPVFDEKHYVPQAWDMVRSWINPITG	86
PMT_Mycosmeg	40	MTAIIITALAAITRFLNLGSPTDAGTPIFDEKHYAPQAWQVLHN-----D	83
PMT_Coryneglu	87	GIESNPGYGLVHPPPLAKQLEALGEWVFGYTPLGWRIMVAIFGTLTIFAI	136
PMT_Mycosmeg	84	GVEDNPGYGLVHPPVVGKQLIAIGEWLFGYNGLGWRFGAVCGVIVMLV	133
PMT_Coryneglu	137	MAIARRLSGSTMTVTFIAGILALADGVLLVSSRFGMLDIFLVFFITAAAWA	186
PMT_Mycosmeg	134	TRIARRISRSTLVGAIAAGLLIADGVSVSSRTALLDVFLVMFAVAAFAC	183
PMT_Coryneglu	187	LIRDHQMHQRLNDLLLTNGQITKD-FGPRFGFRWWRFTTGVFLGLALS	235
PMT_Mycosmeg	184	LMVDRDQVRERMYHAFL-DGRIAETRWGTRLGVRWWRFGAGVLLGLACAT	232
PMT_Coryneglu	236	KWSGLYYIAFFGLTSVFLDLWLKRYGVRRYVTGTLKNDVIPALGSLVII	285
PMT_Mycosmeg	233	KWSGLYFVLFFGVMTLVFDAIARKQYHVPWPGMLRRDLGPAAYVFGLI	282
PMT_Coryneglu	286	PALLYIWSRAWFASETSVYRHAKTDGTITEDSILQLFPESIAGWIHYHI	335
PMT_Mycosmeg	283	PFAVYLASYAPWFASETAVNRY-EVGRSIGPDSILPI-PDALRSLWHYTH	330
PMT_Coryneglu	336	SVLEFHGSLTTSSGHSHPWDSKPWAWLVSGRPILYFSSTDISCDVGG---	382
PMT_Mycosmeg	331	AAVRFHNSLTNADGNHHPWESKPWTWPMSLRPVLYAIDNQ---DVPGCCA	377
PMT_Coryneglu	383	-TCRMIYLFGTPAIWWTVPVILWALWSFFARRSRGYVPLVAFAGFL	431
PMT_Mycosmeg	378	QSCVKAVMLVGTAPAMWFIAPVVLGVALWRTVVRDWRYGAVLVGYMAGFL	427
PMT_Coryneglu	432	PWLAAYDRQMYFFYATALVPFTIIMLALACGELWGRGKMTPTGLTRGSMA	481
PMT_Mycosmeg	428	PWFADIDRQMYFFYATVMAFPLVLAIALILGDILYKPNQNPERRTLGLLT	477
PMT_Coryneglu	482	VVTYISLVMMFLAFSPLFYGFVIPDYVYESLMWFPSWR	520
PMT_Mycosmeg	478	VCFYVALVITNFAWMYPILTGLPISQTTWNLQIWLPSWR	516

A.

Start - End	Observed	Mr (expt)	Mr (calc)	ppm	M	Peptide
4 - 12	1067.5026	1066.4953	1066.4931	2.11	0	K.TIAYDEEAR.R
4 - 13	1223.6019	1222.5946	1222.5942	0.37	1	K.TIAYDEEAR.G
18 - 27	987.5530	986.5458	986.5396	6.21	0	R.GLNSLADAVK.V
58 - 67	1264.5911	1263.5838	1263.5870	-2.53	0	K.EIELEDPYEK.I
79 - 100	2203.1602	2202.1529	2202.1492	1.68	1	K.ETDDVAGDGGTTATVLAQALVR.E
105 - 117	1280.7460	1279.7387	1279.7360	2.10	1	R.NVAAGANPLGLKR.G
196 - 208	1477.7025	1476.6952	1476.6885	4.55	0	K.GYISGYFVTDAER.Q
276 - 283	875.4441	874.4368	874.4409	-4.73	1	K.APGFGRRR.K
349 - 362	1670.7175	1669.7102	1669.7067	2.11	1	R.AEIEIENSDDYDREK.L
379 - 390	1273.6810	1272.6737	1272.6673	5.01	1	K.AGAATEVELKER.K
392 - 399	995.5381	994.5308	994.5308	0.0070	1	K.HRIEDAVR.N

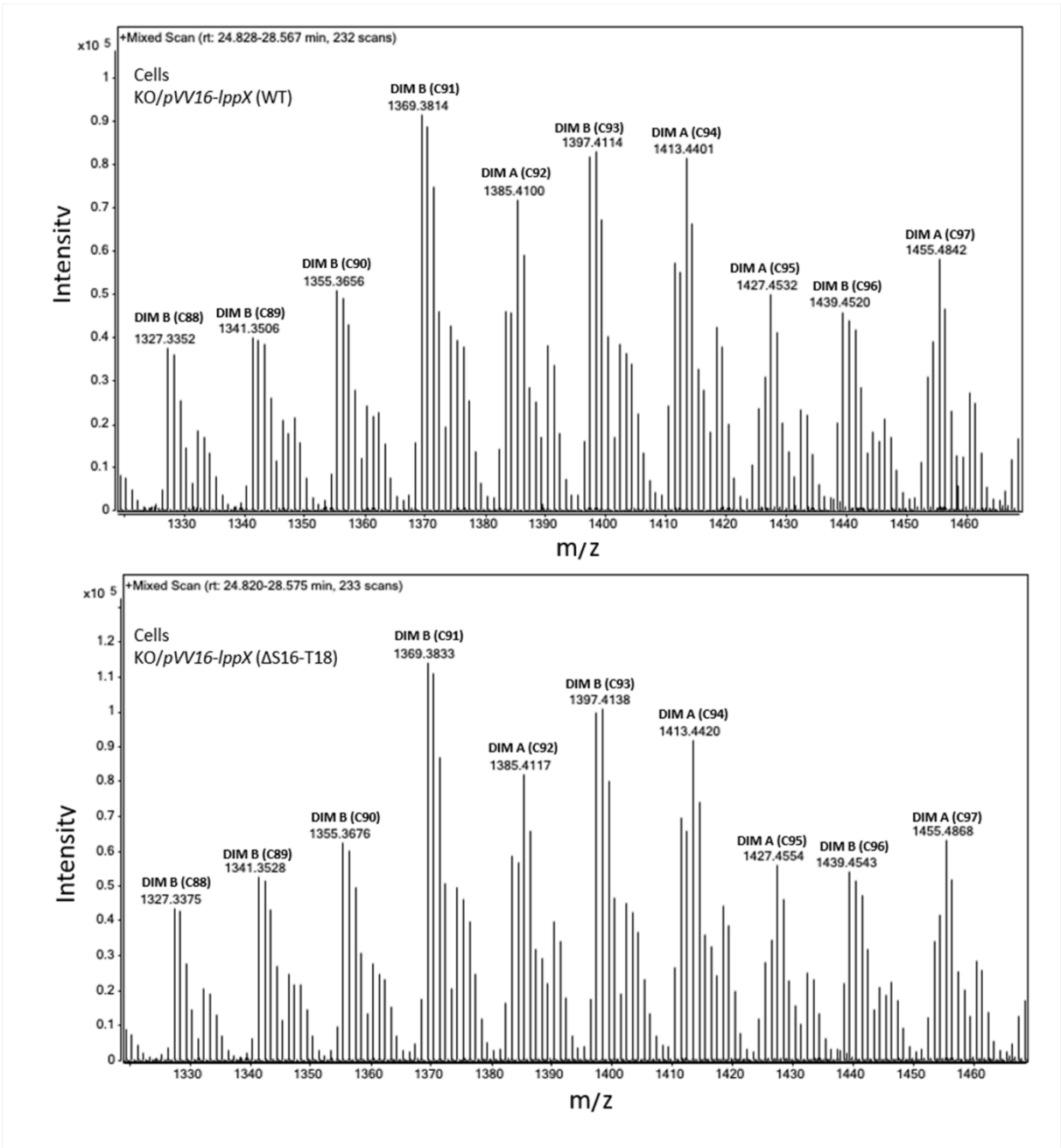
B.

60 kDa chaperonin 1 OS=Mycobacterium smegmatis (strain ATCC 700084 / mc(2)155) OX=246196 GN=groL1 PE=1 SV=1

Protein sequence coverage: 22%

Matched peptides shown in **bold red**.

1 MAKTIAYDEE ARRLERGLN SLADAVKVTL GFHGRNVVLE KFGGAPITN
51 DGVSIAKEIE LEDPYEKIGA ELVEKVAEKT DVVAGDGGTTT ATVLAQALVR
101 EGLRNVAAGA NPLGLKRGIE KAVEKVTETL LKSAKEVETK EQIAATAGIS
151 AGDQSIGDLI AEAMDKVONE GVITVESNT FGLQLELTG MRFDKGYISO
201 YFVTDAERQE AVLEDPYILL VSSKVSIVKD LLPLEKVIQ SGKPLLIIE
251 DVEGEALSTL VVHKIRGTFK SVAVKAPGFG DRRKAMLDQM AILTGGQVIS
301 EEVGLSLETA DVSLLGKARK VVTKDETTI VEGAGDAEAI QGRVAQIRAE
351 IENSDDYDR EKLQERLAKL AGGVAVIKAG AATEVELKER KHRIEDAVRN
401 ANAAVEEGIV AGGGVALLQS AFSLEELSLT GDEATGANIV RVALSAPLKQ
451 IALNGGLEFG VVAEKVSNLP AGHGLNAATG EYEDLLAGV ADPVKVRISA
501 LQHAASIAAL FLTTEAVVAD KFEKAAAPAG DFTGGHGGMD F



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	<i>M. bovis</i>	SIU01591.1	CSSPKPDAAEQGVV SPTAS DPALLAEIRQSLDATKGLTS
	<i>M. leprae</i>	WP_010907555.1	C S STKPDAQEQ SSSSS PASSDPAL T AEIKQSLETTKALSS
	<i>M. marinum</i>	WP_094357885.1	CSSDKTQPEAQ SSTT VPADPALMAEIEQSLDATKAL TSAH
	<i>M. ulcerans</i>	WP_134427244.1	CSSDKTQPEAQ SSTT VPADPALMAEIEQSLDATKAL TSAH
790	<i>M. goodsonae</i>	WP_069434779.1	CASAPKE S EAPPATG TTTT SPLMADVRSV DATKAL TSAH